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PATTERNS OF METABOLIC ACTIVITY DURING AGING OF THE WILD TYPE AND LONGEVITY MUTANTS OF *CAENORHABDITIS ELEGANS*

Bart P. Braeckman, K. Houthoofd and
Jacques R. Vanfleteren
Department of Biology, University of Gent
9000 Gent, Belgium

ABSTRACT

At least three mechanisms determine life span in *Caenorhabditis elegans*. An insulin-like signaling pathway regulates dauer diapause, reproduction and longevity. Reduction- or loss-of-function mutations in this pathway can extend longevity substantially, suggesting that the wild-type alleles shorten life span. The mutations extend life span by activating components of a dauer longevity assurance program in adult life, resulting in altered metabolism and enhanced stress resistance. The Clock (Clk) genes regulate many temporal processes, including life span. Mutation in the Clk genes *clk-1* and *gro-1* mildly affect energy production, but repress energy consumption dramatically, thereby reducing the rate of anabolic metabolism and lengthening life span. Dietary restriction, either imposed by mutation or by the culture medium increases longevity and uncovers a third mechanism of life span determination. Dietary restriction likely elicits the longevity assurance program. There is still uncertainty as to whether these pathways converge on *daf-16* to activate downstream longevity effector genes such as *ctl-1* and *sod-3*.

There is overwhelming evidence that the interplay between reactive oxygen species (ROS) and the capacity to resist oxidative stress controls the aging process and longevity. It is as yet not clear whether metabolic homeostasis collapses with age as a direct result of ROS-derived damage or is selectively repressed by longevity-determining genes. The dramatic decline of protein turnover during senescence results in the accumulation of altered enzymes and in a gradual decline of metabolic performance eventually followed by fatal failure of the system.

To whom all correspondence should be addressed:
Dr. Jacques R. Vanfleteren
Department of Biology
University of Gent
Ledeganckstraat 35
B-9000 Gent
Belgium
Tel: 32-9-2645212
Fax: 32-9-2645344
e-mail: jacques.vanfleteren@rug.ac.be

INTRODUCTION

Poikilothermic animals show an inverse relationship between the duration of each part of the life cycle and ambient temperature. Thus the gradual decline of metabolic activity, which heralds advancing senescence, also proceeds at a slower rate at low temperature. In *C. elegans* the duration of the growth phase and mean life span are about twice as long at 16°C compared to 24°C (Klass, 1977). Coefficients relating the "speed of living" to temperature in poikilotherms, have similar magnitudes. In fact they reflect the acceleration of (bio)chemical reactions as temperature increases. So it would appear that the rates of living and aging are determined by simple underlying thermodynamic processes.

In homeothermic animals, such as mammals and birds, metabolic rate is inversely proportional to body size and life span. In fact, a great number of mammalian species metabolize about the same amount of energy per gram body weight per lifetime (Rubner, 1908) suggesting that organisms have a limited capacity for energy production, possibly because of adverse by-products of metabolic activity. Pearl (1928) combined both observations in his rate-of-living theory which states that there is a species-specific intrinsic maximum to energy production ("metabolic potential") and that life span is inversely proportional to metabolic rate. This hypothesis has been very attractive, particularly since it assumes commonly known processes of wear and tear as underlying causes of accumulating damage and death, although there are many outstanding exceptions. For example, bats, including non-hibernating species, may live much longer than expected from their small body size and fast metabolism (Finch, 1990).

More recently the rate-of-living theory has been revived by incorporating Harman's (1981) free-radical or oxidative damage theory of aging to explain why there is a limit to the energy a species can expend during its lifetime. This theory postulates that aging reflects the accumulation of molecular damage caused by various reactive oxygen species (ROS) that are produced as by-products of oxidative metabolism e.g. superoxide, hydroxyl radicals and hydrogen peroxide. Thus higher metabolic rates would increase oxidative stress, hasten aging and shorten life span. The age-specific decline of metabolic activity could also be ascribed to oxidative injury to the mitochondria in particular, since some 2-4% of the oxygen consumed is diverted to superoxide and derived ROS.

However, life span potential varies over several orders of magnitude among species. The maximum life span of humans is around 120 years, *C. elegans* lives almost 1000-fold shorter. Thus genetic predestination is a key component to longevity. A likely means by which genes could control life span is by specifying the capacity of the cellular defences to prevent or to repair ROS-derived damage. A different way would be by controlling protein metabolism and adjusting protein turnover rates so as to control potential accumulation of altered proteins, particularly enzymes. Thus direct genetic control and oxygen-derived injury are potentially competing or collaborating drives in controlling metabolism in the aging organism. Hence, the essence of understanding aging lies in understanding how metabolic activity is regulated and how genes and ROS interact as age advances.

The Nematode Model System

The use of nematodes to study aging was initiated in the early seventies. The species used included *Turbatrix aceti* (the vinegar eel), *Panagrellus redivivus,* and *Caenorhabditis elegans.* All three species belong to the order Rhabditida and are free-living (they have no parasitic stages in their life history). They combine a number of advantages for the study of aging, including a short life span, small size, ease of cultivation on a single species of bacterium (monoxenic culture), or in synthetic media in the absence of other organisms (axenic culture). They have a relatively small number of cells and, most importantly, no somatic cell divisions occur in the adult worms: old worms contain old somatic cells exclusively. *T. aceti* and *P. redivivus* are dioecious (gonochoristic, separate sexes) species that belong to the family Panagrolaimidae. *C. elegans* belongs to the Rhabditidae and is predominantly monoecious. All three species were explored as potential models for aging research encompassing diverse areas, including life history, behaviour, and ultrastructure. Most work on protein metabolism and enzyme function was carried out by Rothstein and his collaborators and focussed on *T. aceti* (reviews by Rothstein, 1980; 1982). The large amount of knowledge on *C. elegans* in all areas of biology, but particularly in molecular genetics, and which culminated with the recent completion of the 97 megabase genome sequence has stimulated researchers to focus on this single species (The *C. elegans* Sequencing Consortium, 1998). For that reason the *C. elegans* model system will be outlined in much more detail. Additional information can be found in Wood, 1988, Epstein & Shakes, 1995 and Riddle et al., 1997.

C. elegans has a complex mode of reproduction. Most worms are hermaphrodites. As fourth stage larvae (L4) they produce sperm cells which are stored in the spermatheca. Sperm production ceases at the L4 to adult molt and is followed by oogenesis. The oocytes are fertilized as they pass through the spermatheca and an impermeable shell forms around the cleaving eggs in the uterus. Eggs are usually expelled before embryogenesis is completed. A first stage larva hatches within one day at room temperature and passes through 3 more larval stages, all separated by molts. Males arise spontaneously, but at low frequency (0.1-0.2%), following meiotic non-disjunction (hermaphrodites have 2 X chromosomes, males have only one). They can fertilize hermaphrodites, however, and half of the resultant progeny are males. Such male stocks can be maintained by crossing at each subsequent generation. Thus this mode of reproduction allows both selfing and outcrossing as needed for genetic analysis. Thousands of mutants have been isolated and mapped to the six linkage groups (5 AA, XX or X0). Strains are conveniently kept in liquid nitrogen and resuscitated if needed.

C. elegans can be cultured on agar plates carrying a lawn of *Escherichia coli* cells, or on *E. coli* cells suspended in S buffer (0.1 M NaCl, 0.05 M potassium phosphate, pH 6). Nematodes nutritionally require sterol because they lack the capacity for de novo sterol biosynthesis. This is usually met by adding 5-10 µg/mL cholesterol to the nutrient medium. The life cycle takes about 3-3.5 days and mean life span is 2-3 weeks in monoxenic culture and at room temperature (21-22 °C). A convenient axenic culture medium for *C. elegans* is composed of 3% (w/v) dry yeast extract, 3% (w/v) soypeptone and 0.5 mg/mL hemoglobin as a suitable source of heme, which is also an essential nutrient for nematodes. Enough sterol is usually added as a contaminating fraction of the other components. Nematodes may suffer a nutritional deficit in this medium as suggested by slower growth (life cycle of 5-6 days), reduced reproductive output and substantially prolonged life span (4-5 weeks).

Large quantities of age synchronous worms may often be needed for metabolic studies. This can be accomplished by dissolving gravid adults using alkaline bleach (Sulston & Hodgkin, 1988). The eggs released are allowed to hatch overnight in S buffer, and in the absence of food all larvae will arrest at the first stage. Synchronous populations can also be initiated from dauer larvae that are allowed to recover on fresh agar plates seeded with *E. coli.* Offspring must be prevented or removed to avoid intermingling of the adults with their progeny. Small juvenile stages can be removed by sieving, but this method proved effective with *T. aceti* only (Rothstein and Sharma, 1978). Reproduction can be prevented by adding fluorodeoxyuridine (FUdR) to L4 larvae or immediately after the fourth molt. FUdR inhibits DNA synthesis and has a selective detrimental effect on rapidly dividing cells such as embryonating eggs. FUdR treatment has no adverse effects on normal development and morphology when added near maturity and at levels not exceeding 50 µM. Still, this treatment may entail subtle side effects e.g. life span extension by approx. 10%, and mild increases of SOD activity.

Alternatively, genotypes may be used that have a temperature sensitive mutation that induces sterility when the worms are shifted to the restrictive temperature (25 °C). A caveat here is that these mutations may

have unknown pleiotropic effects on aging. One such mutation *fer-15(b26)* extends life span by about 10% in axenic culture (Vanfleteren et al., 1998)

Adult hermaphrodites lay some 300 eggs in about 4 days on lawns of *E. coli* and they continue to produce unfertilized oocytes for some time. Next they enter the postreproductive phase of the life cycle in which the gonads progressively atrophy and various symptoms of senescence appear. The frequency of swimming motions, pharyngeal pumping, and defecating decrease gradually. Shortly before dying worms may become progressively paralyzed, except for rare slow motions of the head. Lipofuscin-like granules that have spectrofluorometric characteristics resembling mammalian age pigment accumulate in the intestinal cells of senescing worms (Zuckerman & Himmelhoch, 1980). This material is currently believed to be derived from subcellular organelles that have been cross-linked as a result of peroxidation of their lipid constituents and that have been encompassed by lysosomes. Accordingly fluorescent material accumulates faster in worms exposed to hyperoxic conditions, and this material and protein carbonyls, also derived from oxidation, accumulate more rapidly in the oxygen-sensitive mutant *mev-1(kn1)* (Adachi et al., 1998; Hosokawa et al., 1994). The alteration of lysosomal enzymes with age is not informative, however. It appears that protease activity declines (Sarkis et al., 1988) and hydrolase activity increases substantially (Bolanowski et al., 1983).

The Dauer Stage has an Altered Metabolism

Under unfavourable conditions of growth *C. elegans* larvae may molt to produce a dauer larva rather than a regular L3 larva. Dauer larvae are much thinner and denser than L3 larvae due to radial shrinkage at the dauer molt. The dauer stage has morphological, physiological and biochemical characteristics that likely have been specifically recruited to enhance survival and dispersal and that are not seen in the other stages. They have an altered cuticle, which is very impermeable and the mouth is closed by a cuticular block. Other contact points with the outside world are modified accordingly. The excretory gland is inactive, contact of sensory organs with the environment is more remote (inner labial sensilla) or likely protected by secreted material (amphid openings). Together, these features provide dauers with an increased resistance to harsh chemical conditions. For example dauer larvae, unlike all other stages, withstand exposure to 1% SDS.

The dauer larva forms in response to limited food supply and high population density. The worms constitutively excrete dauer inducing pheromone, which has chromatographic properties of fatty acids and bile acids. An opposing signal of nucleoside or carbohydrate nature is released by the bacterial cells, and is also present in axenic medium. L1 and L2 larvae and dauers somehow calculate the pheromone to food signal ratio and eventually reach a commitment to molt to the third stage, to form a dauer, to exit from the dauer stage or to remain in the dauer stage. This evaluation is also temperature dependent, with higher ambient temperature favouring the dauer stage. Accordingly, mutations in this pathway can result in the inability to form dauers (Daf-d, dauer defective), or, in contrast, in the production of dauers, even when the environmental conditions favour non-dauer formation (Daf-c, dauer constitutive). Several Daf-c genes are temperature-sensitive (ts): they develop as wild type at low temperature (15°C) and show the mutant phenotype at the restrictive temperature, usually 25°C (comprehensive reviews by Riddle, 1988; Riddle & Albert, 1997).

Dauer larvae can survive for months, at least 8-10 times as long as the mean adult life span. They have been considered to be non-aging because the adult life span is independent of the time spent in the dauer state (Klass & Hirsh, 1976), and they probably die only when they fall short of energy.

Dauer larvae are hyperresistant to environmental stress including elevated temperature (Anderson, 1978) and γ-radiation (Yeargers, 1981), and they have elevated levels of SOD and catalase (Anderson, 1982; Larsen, 1993; Vanfleteren, 1993). Dauer larvae are also 15-fold enriched for heat shock protein 90 mRNA. HSP 90 readily forms complexes with other proteins, such as steroid hormone receptors, generally acting as a negative regulator of transcription (Dalley and Golomb, 1992).

As dauers don't feed they must rely on their energy reserves for survival. Nematodes use lipid and carbohydrate reserves for the production of energy, but lipid is the principal energy reserve in free-living nematodes, and is stored chiefly in the intestinal cells. Plants, microorganisms, and nematodes can use the glyoxylate cycle to transfer two acetyl-CoA units into one molecule oxaloacetate and can thus convert fat to carbohydrate as needed. A key enzyme of this pathway is isocitrate lyase, which cleaves isocitrate into glyoxylate and succinate. The resulting glyoxylate condenses with a second acetyl CoA molecule to malate, a reaction that is catalyzed by malate synthase. In *C. elegans* a single polypeptide contains two distinct structural domains one having isocitrate lyase, the other malate synthase activity (Liu et al., 1995). The glyoxylate cycle has a major role in cleaving eggs and in L1 larvae. In the following stages the relative contribution of the TCA cycle to energy metabolism increases substantially, so that in adults the flux of metabolites through the TCA cycle is much higher than that generated through the glyoxylate pathway. In the dauer stage the flux of metabolites through the glyoxylate cycle is not altered appreciably, but the TCA cycle enzymes are specifically repressed, resulting in a 11.6 fold reduction of the flux of metabolites through that cycle (0'Riordan and Burnell, 1989, 1990). The relative concentration of ATP, ADP, AMP, sugar phosphates and other metabolites produce specific phosphorous nuclear magnetic resonance (NMR) spectra. These spectra change during the life cycle, and they indicate a low energy state in L1 larvae, consistent with high activity of the glyoxylate pathway in that stage relative to

L2-L4. The predominant phosphorous NMR signal in dauer larva extracts corresponds to inorganic phosphate (Wadsworth and Riddle, 1989).

The oxygen consumption rates provide a third line of evidence in support of substantial changes in energy production through the life cycle. If the respiratory rates of embryonating eggs are assigned a value 1, the subsequent developmental stages have the scores 1.52 (L1), 1.67 (L2), 2.93 (L3), 2.86 (L4) 0.94 (3.5-day-old adult), 0.76 (dauer) (Vanfleteren and De Vreese, 1996). In a previous study, using axenic nematodes and a different technology (cartesian diver method), all values were considerably lower and the highest values were obtained for L2 rather than L3 and L4 (De Cuyper & Vanfleteren, 1982). This illustrates how much physiological properties can vary with the environmental conditions.

Free-living nematodes have limited carbohydrate reserves. Dauers effectively metabolize glycogen but their capacity for resynthesis is suppressed. The relative rates of fructose 1,6-bisphosphatase (low levels) and phosphofructokinase (relatively high levels) activity in dauers indicate that the glycolytic pathway is active and that the gluconeogenic pathway is suppressed. The relatively high levels of phosphoenolpyruvate carboxykinase (PEPCK) in these larvae may therefore serve other functions than gluconeogenesis. O'Riordan and Burnell (1989) hypothesized that PEPCK catalyzes the fixation of CO_2 in phosphoenolpyruvate (PEP) in dauers. The resulting oxaloacetate could then be converted to malate by malate dehydrogenase and act as a substrate in the glyoxylate cycle or in the production of succinate in the "reverse" TCA cycle. Indeed, recovery of carbon atoms otherwise lost as CO_2 would constitute a useful anaplerotic mechanism that can enable dauers to survive for months without feeding (O'Riordan and Burnell, 1989).

In the non-dauer stages of *C. elegans* and in *T. aceti* and *P. redivivus*, which lack a dauer stage altogether, PEPCK could be involved in the production of glycerol via enzymes of the glycolytic cycle operating in the reverse direction. The glycerol synthesis path is very active when the worms are maintained in a nutritionally rich medium. It has been proposed that glycerol production, which is predominantly excreted into the culture medium, acts as a mechanism to remove end products of metabolism (Liu & Rothstein, 1976).

It is widely accepted that the combination of the dauer properties of elevated resistance to multiple stress factors and downregulation of metabolic rate account for the long term survival of dauer larvae.

Protein Turnover Rates decrease while Altered Enzymes increase in aging Nematodes

Age dependent declines of enzyme activity have long been thought to underlie the physiological and biochemical deficits that characterize aging. Orgel (1963) argued that low error rates inevitably occur when protein is synthesized. Errors introduced in proteins involved in the protein synthesizing system would tend to generate further errors, potentially leading to a deadly "error catastrophe". In the young, unlike the aged, repair mechanisms would maintain the occurrence of errors well below a threshold level, thus preventing initiation of this feedback mechanism. The error catastrophe theory of aging has stimulated the search for altered proteins considerably in the sixties and seventies. Most of the work on nematodes utilized *T. aceti* and was carried out by Rothstein and his collaborators (reviews by Rothstein, 1980; 1982).

They found several enzymes to be altered in old nematodes including isocitrate lyase (IL, Reiss & Rothstein, 1975), phosphoglycerate kinase (PGK, Gupta and Rothstein, 1976a), enolase (Sharma et al., 1976) and aldolase (Reznick and Gershon, 1977; Goren et al., 1977). A set of solid criteria was used for detection of altered enzymes: (1) specific activity, (2) immunotitration using serum raised against purified young enzyme and (3) heat sensitivity patterns. As pointed out by Rothstein (1980, 1982) the decrease of catalytic ability may result from one of three situations: (1) the "old" enzyme consists of a single type of molecules with reduced catalytic activity, (2) "old" enzyme is a mixture of unaltered plus altered molecules, (3) "old" enzyme consists of molecules with a range of activities. The heat sensitivity patterns and the immunotitration approach (to measure the amount of cross-reacting material present per unit of enzyme) help to distinguish among these possibilities. Interestingly, there was no common pattern by which the aging enzymes underwent alteration, instead, each enzyme altered in a very specific fashion.

In *C. elegans*, the specific activities of tRNA methylases, arginyltransferase and tubulin:tyrosine ligase decrease 5-10-fold in old animals relative to young adults. Sixteen aminoacyl-tRNA synthase activities also decline with age, most of these substantially so, and only the one for tryptophan increases (Gabius et al., 1983). Analogous age-related changes have been detected in mammals as well, including aldolase, phosphorylase, PGK, lactic dehydrogenase, tyrosine aminotransferase and ornithine decarboxylase from various mouse and rat tissues (reviewed in Rothstein, 1982). Other enzymes in nematodes and mammals remain unchanged with age. For example TPI from *T. aceti* isolated from young and old organisms was identical by specific activity, heat sensitivity and immunotitration (Gupta & Rothstein, 1976b).

Thus altered enzymes do occur with age in organisms as diverse as nematodes and mammals. To answer the question as to the structural basis for these changes, Rothstein and his co-workers rigorously investigated a number of additional protein properties, including the K_m values, molecular weight, terminal amino acid residues, charge, gelelectrophoretic pattern, number of SH groups, absence of methionine sulfoxide, immunodiffusion pattern, spectral properties and inactivation by proteases. In fact no sequence changes could be assigned and the experimental evidence thus strongly disproved the idea

of an error catastrophe. Neither did these investigations provide support for the idea of postsynthetic modification as an alternative explanation for the age-related changes in enzymes. This was also confirmed by reports that high resolution 2-D gelelectrophoretic analysis of *C. elegans* proteins detects only few such changes (Johnson & Mc Caffrey, 1985; Vanfleteren & De Vreese, 1994)

The remaining alternative explanation is that altered enzymes result from conformational changes without covalent modifications. Spectral differences between "young" and "old" enolase and PGK disappeared in concentrated guanidine solutions, well in support of this view. Very strong supporting evidence comes from the isolation and identification of an inactive form of enolase, which could be derived in vitro from young enolase. This material precedes the old enolase on an ion exchange (DE32) column, and on rechromatography of the recovered active enolase, some of this material is seen again, apparently being derived from the purified active fraction. An immunological identical product is present in old but not young *T. aceti.* Interestingly successive rounds of rechromatography of "young" enolase also produces this material, and antiserum prepared against thrice columned young enzyme reacts better with "old" enzyme. Similarly, when "young" and "old" enolase are unfolded in guanidine chloride solutions they can refold to form a single population of molecules whether formed from young or old enolase. By a number of criteria the refolded enzyme is very similar, but not identical to "old" enolase, and the heat sensitivity curve is not distinguishable from the thrice columned "young" enzyme. Thus it appears that "young" enzyme may convert to conformational isomers in vitro and in vivo (Sharma & Rothstein, 1978a; 1978b; 1980).

Why are these altered enzymes seen in old but not young animals? Rothstein and his co-workers argued that this results from a slowed protein turnover in aged worms, and they advanced experimental evidence strongly supporting this hypothesis. In short, worms were fed labelled amino acids (^{3}H Leucine or ^{35}S Methionine) for 12-24 hr, washed and unincorporated label was chased by incubating them in medium containing cold amino acids. The decrease in the amounts of labelled protein was then followed to obtain the half life values ($t_{1/2}$), the times needed to reduce the label by 50%. Complex soluble protein fractions and the purified single proteins enolase and aldolase were studied. These experiments demonstrated unequivocally that there is a dramatic slowing of protein turnover with age in *T. aceti.* For example $t_{1/2}$ values for soluble proteins increased about 10-fold in 20-day-old relative to 2-day-old worms (Prasanna and Lane, 1979). As a direct result of the slowing of protein turnover, the individual life spans of the protein molecules, or "dwell times" increase substantially, and they have ample time to undergo structural alteration, and to accumulate instead of being replaced with the intact molecules. The precise causes of these subtle conformational changes are not known, but they may simply reflect intrinsic thermodynamic instability (Rothstein, 1980, 1982).

Attempts have been made to relate the slowing of protein turnover rates in aging nematodes to defects in ribosomal and lysosomal function, but these studies have mostly led to confusion and controversy.

Mutations Affecting Diapause and Longevity Define an Insulin-like Signaling Pathway in *C. elegans*.

More than 30 genes controlling dauer larva formation have been identified and ordered into branched complex pathways (Riddle & Albert, 1997). A subset of these genes also affect longevity and form a terminal branch of the pathway. The Daf genes involved include *daf-2, age-1* (both Daf-c), and the Daf-d genes *daf-12, daf-16* and *daf-18.*

The first life span mutants isolated identified the gene *age-1.* Two reduction-of-function alleles *hx542* and *hx546* (now believed to be identical) extend life span by 65% without having adverse effects on development, mortality or fertility (Friedman & Johnson, 1988a, 1988b). Quite similar phenotypes were later observed for mutants in *daf-23* (Daf-c). This gene was cloned and found to encode a homologue of mammalian phosphatidylinositol 3-kinase p110 catalytic subunits (Morris et al., 1996). Complementation studies eventually demonstrated that *daf-23* and *age-1* are allelic (the annotation *age-1* being retained). However, the open reading frame of *age-1(hx546)* is intact suggesting that the mutation *hx546* might rather affect a closely linked promotor or enhancer element. This might also explain the very weak Daf-c phenotype of *age-1*, which becomes only apparent at 27°C and was not known initially (Tissenbaum & Ruvkun, 1998).

Reduction of function mutations in *daf-2* (Daf-c) can also extend longevity substantially. For example *daf-2(e1370)* mutant worms live twice as long at 25°C (Kenyon et al., 1993). Because the *e1370* mutation is a ts Daf-c mutation, growth must be initiated at the permissive temperature to prevent dauer arrest. The worms can be shifted to the restrictive temperature after they have molted to the L3 stage. It should be stressed that mutations that reduce the function of both *age-1* and *daf-2* prolong survival, so that wild-type alleles of these genes act to shorten life span.

Interestingly, mutation in *daf-16* can completely suppress the phenotypes imparted by *age-1* and *daf-2.* Thus intact activity of *daf-16* is required for the extension of life span dictated by mutation in *daf-2* and *age-1* to occur. The very small life span shortening effect of mutation in *daf-16* by itself can by no means account for the decrease in life span seen in the doubles with *age-1* and *daf-2.* Mutation in *daf-12* also causes a small decrease in life span, but *daf-2(e1370); daf-12(m20)* double mutants live four times longer than wild type when shifted to the restrictive temperature after completion of the second molt. When incubated at the restrictive temperature directly after hatching these larvae fail to form dauers, but arrest as young larvae. It should be

added that there is considerable complexity of epistatic interactions, caused by a wide range of allele-specific effects (Larsen et al., 1995) and by the bifunctional (denoted A and B) character of *daf-2* and *daf-16*. For example, Gems et al. (1998) provided evidence that *daf-12* specifically interacts with *daf-2*A and *daf-16*A to promote dauer larva development and increase adult longevity.

The molecular characterization of these genes indicates that they all encode components of a single signaling pathway. *daf-2* encodes a member of the family of insulin receptor tyrosine kinases (Kimura et al., 1997). *daf-18* encodes a homologue of PTEN, a human tumor suppressor with PI-trisphosphate phosphatase activity (Ogg & Ruvkun, 1998; Rouault et al., 1999; Mihaylova et al, 1999). Two novel genes, *akt-1* and *akt-2*, discovered in a suppressor screen for loss of function alleles of *age-1*, are homologues of mammalian serine/threonine kinase Akt/PKB (Paradis & Ruvkun, 1998) which are known to transduce signals from PI-3 kinase. RNAi studies showed that *akt-1* and *akt-2* function in a redundant fashion to prevent dauer formation, but the precise function of both genes in the signaling pathways emanating from *daf-2*, and those regulating aging in particular, remains to be solved.

Two loss-of-function and one gain-of-function allele of the gene *pdk-1* were discovered in a screen for novel mutants that would arrest as dauers at 27°C. PDK-1 is a nematode homologue of the mammalian Akt/PKB kinase PDK1. The major function of PDK-1 is to transduce signals from AGE-1 PI-kinase to AKT-1 and AKT-2 (Paradis et al., 1999). Finally, *daf-16* encodes two gene products which are produced by differential splicing, and belong to the forkhead family of transcription factors (Ogg et al., 1997; Lin et al., 1997; Paradis & Ruvkun, 1998), and *daf-12* encodes a nuclear hormone receptor of the steroid/thyroid receptor superfamily (Yeh, 1991).

Most recently Ailion et al. (1999) reported on 2 genes that control both life span and dauer formation and likely function upstream in the insulin receptor pathway. Mutations in *unc-64* and *unc-31* increase life span and cause constitutive dauer formation at 27°C. Both the Daf and Age phenotypes of an *unc-64;unc-31* double mutant are stronger than those of either single mutant and both are suppressed in *daf-16*. Thus *unc-64* and *unc-31* function in the insulin-like pathway and are partially redundant. *unc-64* encodes a homologue of syntaxin, a protein involved in synaptic transmission; *unc-31* encodes a homologue of Ca^{2+}-dependent activator protein for secretion (CAPS), a protein required for Ca^{2+}-stimulated peptide secretion from a mammalian neuroendocrine cell line, and the expression of these two genes coincides only in neurons. Based upon these data, Ailion and his collaborators suggest that *unc-64* and *unc-31* mediate secretion within the nervous system, either directly or indirectly, of an insulin-like ligand for the DAF-2 receptor.

A plausible model (Fig.1) that integrates all genetic and molecular data proposes that *daf-16* acts as a repressor of genes that regulate prosperous growth and abundant reproduction. When the *daf-2/age-1* signaling pathway is activated, DAF-16 is phosphorylated and loses its repressor function, or may acquire an activator function instead, and allow the expression of the metabolic genes necessary for reproductive development and short life span (Guarente et al., 1998). Under favorable growth conditions an as yet unidentified insulin-like ligand binds to DAF-2 receptorkinase. DAF-2 in turn phosphorylates itself and recruits and activates PI 3-kinase, which then generates second messenger(s). 3'-Inositide second messenger activates PDK-1 which activates AKT-1 (it is thought that AKT-2 may transduce DAF-2 signaling independently of PI 3-kinase) which in turn regulates DAF-16, most likely by means of a phosphorylation cascade. When the conditions are unfavourable, DAF-2 is not activated, the signaling cascade is off and DAF-16 is constitutively active, causing a shift to dauer arrest, energy storage, dauer-specific metabolism, enhanced stress resistance and extended survival (Guarente et al., 1998; Paradis & Ruvkun, 1998; Tissenbaum & Ruvkun, 1998; Paradis et al., 1999).

It remains to be shown how the nuclear hormone receptor DAF-12 interacts with the insulin-signaling pathway. Daf-c mutations in *daf-2* and *age-1* have antagonistic activity with *daf-12* (Gottlieb & Ruvkun, 1994; Larsen et al., 1995). However, the Age phenotype is strongly enhanced in some *daf-2;daf-12* doubles. One possibility is that mutations in *daf-12* might suppress a presumed pleiotropic effect of the *daf-2* mutations. An alternative explanation is that DAF-12 acts as a receptor for secondary signals released when the signaling cascade is activated. These secondary signals may then interact with DAF-12 to shorten or to lengthen life span in an allele dependent fashion. Apfeld and Kenyon (1998) recently demonstrated, by means of mosaic analysis, that *daf-2* functions non-autonomously in dauer formation as well as life span determination. They discovered that a fraction of *daf-2*(-) cells was sufficient to prolong life span of the animal, and that *daf-2*(-) cells can prevent *daf-2*(+) cells from dying, and that there was no evidence of any particular cells or tissue promoting aging. Since DAF-2 is a membrane-bound protein, it is presumed to act by generating secondary signals.

Changes in Metabolism and Physiology of Long-lived Daf-c Mutants are Reminiscent of a Dauer Longevity Assurance Program

Aging *age-1* adults are dauer-like in being hyper-resistant to atmospheric oxygen (Adachi et al., 1998), H_2O_2 (Larsen, 1993) and Paraquat (Methylviologen, generates superoxide anions after reduction by cellular NADPH, Vanfleteren, 1993). *age-1* and *daf-2* adults also exhibit elevated levels of catalase and SOD activity that are likely responsible for the increased resistance against all three forms of oxidative stress. Interestingly these mutants produce increasing antioxidant enzyme

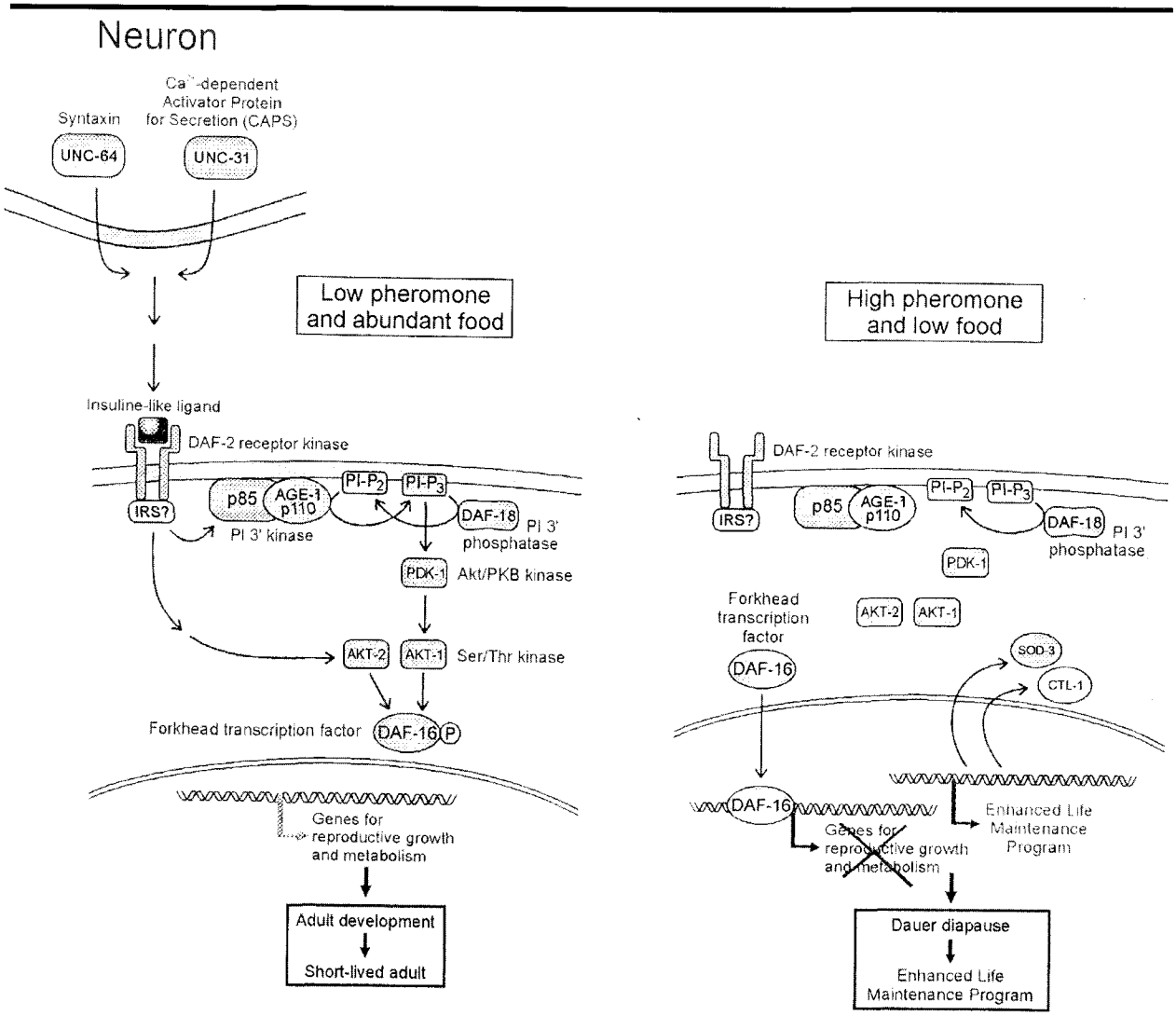

Figure 1: Model for the regulation of life span extension by the insulin-like signaling pathway. This model assumes that DAF-16 acts as a repressor of genes that promote prosperous growth and reproduction, followed by rapid senescence and death. Under permissive conditions of low dauer-inducing pheromone and high abundance of food, UNC-64 syntaxin and UNC-31 CAPS proteins mediate secretion of insulin-like ligand within the nervous system. This ligand binds to DAF-2 receptor kinase and activates a signaling cascade resulting in phosphorylation of DAF-16. Phosphorylated DAF-16 looses its repressor function or may even acquire an activator function, and now allows expression of the genes required for rapid and abundant reproduction and short life. Under conditions of low food and high concentration of dauer pheromone, the signaling cascade is off, non-phosphorylated DAF-16 represses transcription of the genes for reproductive growth and metabolism, and allows expression of the dauer diapause program. Dauer larvae have an altered morphology, their metabolism is shifted from high energy production and usage to energy storage, and they activate a longevity assurance program that protects against oxidative and thermal stress. The oxidative stress defense genes *sod-3* and *ctl-1* are likely components of this program. Long-lived Daf-c mutants express components of the extended survival program, including *sod-3* and *ctl-1*, during adult life.

activities with advancing age (Larsen, 1993; Vanfleteren, 1993; Vanfleteren & De Vreese, 1995). Cu/Zn SOD isoforms account for most of the increase. Cyanide-resistant SOD activity, assayed by monitoring the reduction of Nile Blue tetrazolium in the presence of 10 mM KCN was very low, representing approx. 2% of total SOD activity, but was significantly higher in three *age-1* strains relative to the control strain. An apparent increase in three-week relative to 1-week-old worms observed in all strains was mistrusted because it could be contributed by *E. coli* cells present in the small fraction (about 1%) of dead worms that could not be removed by repeated washings (Vanfleteren, 1993).

There are multiple genes encoding SOD in *C. elegans*. *sod-1* encodes cytosolic Cu/Zn SOD, and was the first to be identified (Larsen, 1993; Giglio et al., 1994a). *sod-4* encodes 2 isoforms of Cu/Zn SOD by differential splicing, one extracellular (SOD4-1) and one membrane-bound (SOD4-2) form (Fujii et al., 1998). *sod-2* and *sod-3* encode mitochondrial Mn SOD (Giglio et al., 1994b; Suzuki et al., 1996; Hunter et al., 1997). Tawe et al. (1998) identified four genes that were differentially

upregulated in larvae as a response to oxidative stress due to Paraquat. The steady-state mRNA level of glutathione S-transferase, was induced 40-fold and the mRNA levels of Mn SOD and Cu/Zn SOD increased two-fold in the stressed larvae. The *sod-3* gene is differentially expressed in the dauer stage and remains silent in the other stages (Honda & Honda, 1999), suggesting that it is a key component of the dauer longevity assurance program. Interestingly, *sod-3* mRNA is constitutively transcribed in all stages of *daf-2(e1370)*, and is likely responsible for the oxidative stress resistance (Oxr) phenotype observed in the L2 and all later stages. Wild-type alleles of *daf-16* and *daf-18* are required for this effect to occur, suggesting that the insulin-like pathway regulates *sod-3* expression. It is thus possible (though not proven) that the higher Mn SOD activities in *age-1* strains recorded by Vanfleteren (1993) are due to *sod-3*. Both the level of *sod-3* mRNA and the Oxr phenotype are significantly enhanced in *clk-1(e2519) daf-2(e1370)* double mutants, although the *clk-1* single mutant does not express *sod-3*, nor displays the Oxr phenotype by itself (Honda & Honda, 1999). The Age (Lakowski & Hekimi, 1996) and ATP (Braeckman et al., 1999) phenotypes of *clk-1 daf-2* display similar synergistic effects.

C. elegans has two genes encoding catalase. *ctl-2* encodes a peroxysomal catalase that is constitutively expressed in all stages. In adult worms, declining levels of *ctl-2* mRNA are produced as the worms age, however. *ctl-1* codes for an unusual cytoplasmic isoform, that is also expressed in all stages. Unlike *ctl-2, ctl-1* is strongly overexpressed in the dauer stage and in *age-1* (and the other Daf-c long-lived mutants presumably as well) adults (Taub et al., 1999). *ctl-1* is thus a second key component of the dauer longevity assurance program, and the altered catalase profiles recorded for the *age-1* strains by Larsen (1993) and Vanfleteren (1993) likely reflect additional expression of *ctl-1*.

The loss-of-function mutation *ctl-1(u800)* reduces total catalase activity by more than 50% and shortens life span by 23%, relative to wild-type animals, and eliminates the life span extension conferred by mutation in *age-1* or *daf-2*, suggesting that *ctl-1* is downstream of these genes, and that it is required for the life span extension conferred by these mutations. This epistasis is only seen for life span and not for dauer formation. The expression of *ctl-1* may be directly regulated by *daf-16*, so that inappropriate upregulation of *daf-16* in daf-c longevity mutants would also confer upregulation of *ctl-1* (Taub et al., 1999).

age-1 and *daf-2* adults also show increased intrinsic thermotolerance (Itt) (Lithgow et al., 1994; 1995), possibly caused by their overexpressing HSP-16 (Lithgow, 1996). The increase of stress resistance was later found also to cover UV stress, and in fact it was soon realized that all existing long-lived mutants are resistant to UV stress (Uvr phenotype), and most of them are resistant to all three forms of stress (Murakami and Johnson, 1996). Overexpression of *tkr-1* in transgenics confers the Itt, Uvr and Age phenotypes (Murakami & Johnson, 1998). Pretreatment of both wild-type and mutant worms at sublethal temperature induces significant increases in life span (Lithgow et al., 1995). Thus it appears that there is a true causal relationship between ability to resist stress and life expectancy in *C. elegans*. The observation that the Age and Itt phenotypes are positively correlated in a series of 15 *daf-2* alleles further substantiates this point (Gems et al., 1998).

Isocitrate lyase and malate synthase are increased in *age-1* and *daf-2* adults, and both acid and alkaline phosphatase are decreased, as normally occurs in the dauer stage. However, the TCA cycle enzyme isocitrate dehydrogenase (ICD), which is typically repressed in the dauer stage, is not downregulated in these mutants. In addition, the mutant worms retain almost constant levels of ICD activity, whereas ICD declines steadily with age in worms having wild-type alleles of *age-1* and *daf-2* (Vanfleteren & De Vreese, 1995). Reduction of ICD heralds the shift to low energy metabolism in the dauer state, and is accompanied by reduced respiration. A similar shift does not occur in *age-1* and *daf-2* adults, however. The respiration rates do not differ in young *age-1* adults and wild-type animals, and start declining similarly with age as the worms grow older, but after 8.5 days of age the mutant worms retain significantly higher respiratory activity. Thus the ICD profiles and respiration rates militate against the idea that the Daf-c longevity mutants may have lower metabolic rates, but they do suggest that mutant worms of the same age are biologically younger (Vanfleteren & De Vreese, 1996).

Unfortunately respiration assays are not very sensitive. Vanfleteren and De Vreese (1995) proposed an assay which estimates the amount of light that can be produced by freeze-thawed tissue in the presence of lucigenin and plentiful supply of reducing power as NADH and NADPH. KCN is added at 5 mM final concentration in this assay to suppress endogenous Cu/Zn superoxide dismutase activity in the most recent version of this assay (Vanfleteren et al., 1998). Light is emitted when superoxide reacts with lucigenin, after it has been first reduced by endogenous reductases (Faulkner & Fridovich, 1993). Reduced lucigenin can also react directly with molecular oxygen and produce superoxide (Liochev & Fridovich, 1997). Lucigenin can thus act as a source of and a detector of superoxide and the fraction of lucigenin luminescence contributed by autooxidation is essentially unknown. However, the excellent correlation of lucigenin luminescence with oxygen consumption by wild-type and *age-1* mutant worms over their entire life cycle (Vanfleteren & De Vreese, 1996) and the fact that enzymatic reduction of lucigenin to the univalently reduced form is a prerequisite for luminescence, demonstrates that the light production assay provides a reliable estimate of the potential of nematode tissue for metabolic activity immediately prior to freeze fixation. This parameter, which measures the maximal metabolic output, has been called light production potential, superoxide production potential, metabolic capacity and metabolic potential. Note that

"metabolic potential" thus defined is quite different from Rubner's (1908) definition (total amount of energy expended per gram of body weight during the life span).

The metabolic capacity is similar in all wild-type and *age-1* larval stages and in young adults up to age 5-6 days. As the worms grow older the metabolic capacity drops steeply in wild-type, but not *age-1,* worms. The metabolic capacities are similar in wild-type and mutant dauers and are five-fold and two-fold lower relative to the L3 larvae and 3.5 day-old-adults, respectively. Thus neither respiration rate nor metabolic capacity are set at dauer-like levels in *age-1* or *daf-2* adults (Vanfleteren & De Vreese, 1995; 1996). Vanfleteren et al. (1998a) monitored the metabolic capacity and tyrosine kinase (PTK) activities as a means to study interactions between *age-1, daf-2,* and *daf-12.* The single mutants and *age-1; daf-2*, and *daf-2;daf-12* double mutants, and a control strain having wild-type alleles of *age-1, daf-2* and *daf-12* were studied. The metabolic capacity and PTK activities decrease similarly in *daf-12* and the control strain, which is not surprising since *daf-12* has also little effect on life span by itself. The age-dependent decline of the metabolic capacity and PTK activity is significantly reduced in *age-1* and *age-1;daf-2,* and virtually absent in *daf-2* and *daf-2;daf-12* mutant worms. This can be explained assuming that (a) *daf-2* is a major effector of metabolic activity during adult life, and that wild-type alleles of this gene downregulate metabolic activity with increasing age, thus shortening life span; (b) *daf-12* is a minor effector of metabolic activity, and acts independently of *daf-2.* A substantial reduction of the wild-type activities of both genes at the same time would result in a drastic reduction of metabolism and prevent its age-dependent decrease. Alkaline phosphatase activities were also measured in this study and they support these conclusions. However these results are less reliable, since even very small amounts of contaminating *E. coli* cells can provide measurable quantities of alkaline phosphatase activity.

The PTK activity settings are higher in the dauer state relative to L3 larvae and they are similarly upregulated in *age-1* and *daf-2* adults relative to wild-type worms. PKA activities are also higher in dauers relative to L3 larvae. They are downregulated in *age-1* young adult worms, relative to wild type whereas *daf-2* and wild-type worms of the same age exhibit similar activities. Finally, PKC activities are not reduced in young *daf-2* and *age-1* adults, relative to the control as would be expected if a dauer-like setting were followed (Vanfleteren & De Vreese, 1997).

In short, there is a great deal of evidence suggesting that a number of physiological and metabolic activities are set at dauer-specific levels in *age-1* and *daf-2* adults, including components of a dauer longevity assurance program. Other aspects are not so regulated, however. Thus distinct elements of the dauer metabolism seem to be recruited individually for heterochronic expression in these mutants.

Clk Mutations affect the Rate of living and Life Span

The Clk genes constitute a set of genes that control the timing of a wide range of physiological processes. The genes *clk-1, clk-2* and *clk-3* were identified through a screen for maternal effect mutations affecting the time of worm development and behavior (Wong et al., 1995; Lakowski & Hekimi, 1996) in an attempt to uncover genes that might function as timing devices coordinating temporal features of the organism. The fourth gene *gro-1* was originally identified on the basis of its slow growth (Hodgkin & Doniah, 1997) and was later classified with the clock genes on the basis of shared features that define the clock phenotype. These include mean increases of the length of the cell cycle, embryonic and postembryonic development and adult life span and decreases of the rate of behavioral activity patterns, including pharyngeal pumping, defecating, egg laying and moving. In fact the essential defect appears to be a deregulation of the timing of these activity patterns, as suggested by the substantial increase in variability among animals. Occasionally, mutant embryos may even develop faster than wild-type embryos. Alternatively, the increase in variability might reflect variable penetrance. The duration of embryonic development of the clock mutants depends on the temperature experienced up to the two-cell stage i.e. the temperature at which their mothers were maintained, rather than the actual experimental temperature. This would suggest that temperature dependence of metabolic processes in *C. elegans* is an active and adaptive process rather than a mere thermodynamic consequence. Finally all Clock mutations are subject to maternal rescue i.e. homozygous mutant offspring are phenotypically wild type when derived from a heterozygous mother (Wong et al., 1995).

Single Clock mutants show moderate increases of life span. The increases of the adult life span are smaller because of the extra time needed to reach adulthood. Certain double mutants may have total life spans 2-3 times as long as wild-type worms and *clk-1(e2519) daf-2(e1370)* double mutants live almost five times as long (Lakowski & Hekimi, 1996; Wong et al., 1995). There has been much controversy as to whether mutation in *daf-16* suppresses the life span extension seen in the Clock mutants. Lakowski & Hekimi (1996) reported that *daf-16(m26)* failed to suppress mutation in *clk-1.* This would suggest that the Clock and the Daf-c gerontogenes do not converge on *daf-16,* and that they affect life span by fundamentally different mechanisms. However Murakami & Johnson (1996) found essentially similar adult life spans for N2 and double mutants of *clk-1(e2519)* and either *daf-16(m26)* or *daf-16(m27).* The interpretation of the life span of *clk-1;daf-16* double mutants is complicated by the fact that the mutations in *daf-16* slightly shorten life span, relative to the wild type, and this effect tends to obscure the failure of *daf-16* to suppress *clk-1* (Lakowski & Hekimi, 1998). Life spans are generally about 2 times longer in axenic medium and the effects of interacting genes on life expectancy are

Table 1: Life span of *C. elegans* in axenic culture (days)

Exp	*Strain*	*Additives*	*Temp*	*N*	*Mean ± SE*
1	N2	FUdR*	24	59	32.5 ± 0.7
1	*mev-1(kn1)*	FUdR*	24	62	26.4 ± 0.6
1	*rad-8(mn163)*	FUdR*	24	64	27.8 ± 0.4
2	N2	FUdR*	24	62	37.0 ± 1.0
2	*gro-1(e2400)*	FUdR*	24	62	52.8 ± 1.1
2	*fer-15(b26) age-1(hx546);gro-1(e2400)*	FUdR*	24	60	63.8 ± 1.4
2	*fer-15(b26) age-1(hx546);daf-16(m27)*	FUdR*	24	63	38.3 ± 1.2
3	N2	FUdR* / *E. coli***	24	64	30.9 ± 0.8
3	*clk-1(e2519)*	*E. coli***	24	60	63.4 ± 1.4
3	*clk-1(e2519) daf-2(e1370)*	*E. coli***	24	62	75.5 ± 1.5
3	*clk-1(e2519);daf-16(m27)*	*E. coli***	24	59	35.9 ± 1.0
4.	N2	FUdR* / *E. coli***	24	213	26.7 ± 0.4
4	*daf-16(m26)*	FUdR* / *E. coli***	24	209	23.4 ± 0.4
4	*daf-16(m27)*	FUdR* / *E. coli***	24	210	23.9 ± 0.4
5	N2	FUdR*	24	99	25.9 ± 0.6
5	*eat-2(ad465)*	FUdR*	24	110	35.7 ± 0.6

* FUdR was added at 50 µg/ml final concentration

** Autoclaved *E. coli* cells were added at $9x10^5$ cells/ml

enhanced accordingly. *clk-1(e2519)* fails to reach adulthood in standard axenic medium and this defect is not suppressed by *daf-16(m26)* nor *daf-16(m27)*. When autoclaved *E. coli* cells are added to this medium all genotypes grow to the adult stage and *clk-1(e2519)* lives twice as long as N2 (Table 1). The double mutant *clk-1(e2519);daf-16(m27)* retains a weak increase of life span. The life span shortening effect of mutation in *daf-16* in this medium is approx. 10%. Thus mutation in *daf-16* can largely suppress life extension caused by mutation in *clk-1* in axenic culture. This would suggest that the Clk and insulin-like pathways of life extension do converge on *daf-16* and it is in keeping with the need of intact CTL-1 for *clk-1* and *daf-2* and *age-1* life extension (Taub et al., 1999).

Mutation in Clk Genes Affects Energy Consumption Rather than Energy Production

Of the four Clk genes only *clk-1* has been cloned as yet. The encoded protein is structurally very similar to the yeast metabolic regulator Cat5p/Coq7p and its human and murine homologues (Ewbank et al., 1997). In yeast this protein is necessary for the synthesis of ubiquinone, for the release from glucose repression, restoration of oxidative metabolism and gluconeogenic gene activation (Proft et al., 1995; Marbois & Clarke, 1996). Jonassen et al. (1998) demonstrated that cat5/coq7p is a yeast inner mitochondrial membrane protein that is required for the synthesis of coenzyme Q and that all defects seen when the encoding gene is inactive derive from lack of ubiquinone. Although cat5/coq7p and the homologous CLK-1 proteins are structurally well conserved, they have likely functionally diverged. While *clk-1* is able to rescue cat5p/coq7p deficiency in yeast, neither loss-of-function or overexpression of cat5p/coq7p affects yeast longevity (Hekimi et al., 1998). In addition null mutations of *clk-1* only cause mild reduction in respiration in the nematode. Thus it is unlikely that *clk-1* is absolutely required for CoQ biosynthesis in nematodes (Felkai et al., 1999).

In view of the general decline of the rate of temporal functions in Clk mutants it has been hypothesized that *clk-1* mutations affect the rate of metabolism. A reduced metabolic rate would then result in slower production of reactive oxygen species and slow down the aging process (Lakowski & Hekimi, 1996; Ewbank et al., 1997; Hekimi et al., 1998; Felkai et al., 1999). This is the updated rate-of-living hypothesis (Sohal, 1986) which recruits the oxidative damage theory to revive Pearl's (1928) hypothesis of an inverse relationship between metabolic rate and life span. However, respiration is only slightly reduced in the Clk mutants, as measured by direct respiration assays (Braeckman et al., 1999), a mitochondrial dye uptake assay, and by assaying succinate cytochrome c reductase (Felkai et al., 1999). The single Clk mutants examined, *clk-1* and *gro-1*, exhibit metabolic capacities that are only very slightly reduced during the first 4-5 days of their adult lives. At older ages the Clk mutants retain higher metabolic capacities, suggesting that they are biologically younger, than wild type worms. Finally ATP levels are not reduced in these mutants; in *gro-1* mutants they are even higher. In wild-type worms ATP stores decrease exponentially with age after the first 2-4 days of adult life. This decrease is much weaker in Clk mutants. The simultaneous presence of a mutation in *daf-2* or *age-1* enhances this difference dramatically. In fact the ATP levels in *clk-1 daf-2* and *age-1;gro-1* exceed those in wild-type worms and they remain nearly constant with age. Surprisingly, mutation in *daf-16* suppresses the ATP phenotype of the Clk mutants i.e. reduces the ATP levels back to wild-type levels, but not the weak reduction of oxygen consumption (Braeckman et al. 1999)

These experiments do not support the idea that the limitation of energy metabolism is the primary defect, rather they suggest that energy production is uncoupled from energy consumption in these mutants. In an updated model Felkai et al. (1999) propose that wild type CLK-1 functions to adjust nuclear gene expression to mitochondrial activity. In the Clk mutants the nucleus 'believes' that mitochondrial activity is depressed, and downregulates gene expression accordingly, and the rate-of-living is reduced in a coordinated and physiologically acceptable way.

The idea that specific genetic pathways control aging has recently been challenged by Van Voorhies and Ward (1999). These authors measured the CO_2 output produced by 50 adult worms crawling on a lawn of heat-killed bacteria that was spread over an agar surface in small vials. Four strains were studied: (1) N2 (the reference wild-type strain), (2) *age-1(hx546) fer-15(b26)*, (3) *daf-2(e1370)*, and (4) *clk-1(e2519) daf-2(e1370)*. The amount of CO_2 in the gas phase was measured using a CO_2 analyzer. The authors found that CO_2 production of wild-type worms is negatively correlated with temperature. At 20 °C the CO_2 outputs of the long-lived strains were reduced relative to wild type (N2) in the order N2 > *age-1* > *daf-2* > *clk-1 daf-2*. Guided by these observations they concluded that the increased longevity of the mutants is a consequence of a reduction in their metabolic rate, rather than an alteration in a specific longevity program.

There are several methodological problems with this study, however. Firstly, CO_2 output is expressed per individual rather than being scaled to biomass or protein content. However, the strains examined have widely differing body masses. For example, wild-type worms are twice as big as *clk-1 daf-2* mutants. When the data are scaled to body mass the overall picture changes dramatically. Secondly, no attempt was made to account for biases resulting from differences in the number of embryonating eggs in the gravid adults and in the length of the reproductive period, among strains. Thirdly, the correlation of metabolic rate, traditionally defined as oxygen consumption rate, and CO_2, can be biased by changes in metabolic activity, including the nature of the metabolized substrate (fat, carbohydrate, protein), the level of oxaloacetate production by PEPCK-catalyzed fixation of CO_2 in PEP, and the energy flux through the glyoxylate pathway.

The Reduced Life span of Oxygen-sensitive Mutants: Still a Badly Understood Relationship

Although the (weak) reduction of mitochondrial energy production is not the major cause of the life span extension seen in Clk mutants, they admittedly do produce less superoxide and this could have an additional beneficial effect on life span in terms of the oxidative damage theory of aging. In fact, overexpression of *clk-1* leads to an increase in the rate of respiration, acceleration of behavioral rates and reduced life span (Felkai et al., 1999). Mutation in *mev-1* and *rad-8* cause hypersensitivity to raised oxygen concentrations and shorten life span (Ishii et al., 1990; Ishii et al., 1993). The *rad-8* mutant was originally isolated on the basis of its hypersensitivity to UV irradiation (Hartman & Herman, 1982). This mutant is also hypersensitive to high oxygen concentration and to Methylviologen (Ishii et al., 1993), and has an extended life span at low temperature, which is due to its slower development, relative to wild type.

The *mev-1(kn1)* mutation increases the sensitivity to Methylviologen (Paraquat) four-fold and shortens mean life span by 30% (Ishii et al., 1990). Cu/Zn SOD activity is reduced by 30-50% in this mutant, explaining its sensitivity to Methylviologen (Ishii et al., 1990; Ishii et al., 1994; Adachi et al., 1998). This mutant accumulates protein carbonyls at a faster rate than the wild type under atmospheric oxygen and its sensitivity to oxygen is enhanced in a concentration dependent fashion, when exposed to hyperoxic conditions. In contrast, Daf-c long-lived mutants accumulate protein carbonyl at a much slower rate, in a mirror image of the order of their life spans, and they are quite resistant to hyperoxic conditions (Adachi et al., 1998; Yasuda et al., 1999). Protein carbonylation is a good marker of protein oxidation, and these observations suggest that it is also a good marker of physiological age. However it remains unclear whether protein carbonylation is a cause or a consequence of aging.

The *mev-1* gene was cloned and found to encode cytochrome b_{560} which is a component of succinate-ubiquinone reductase (mitochondrial complex II) (Ishii et al., 1998). The mutation *kn1* substitutes glutamic acid for glycine at position 71, thereby reducing complex II activity by more than 80% relative to wild type. Ishii et al. (1998) hypothesize that cytochrome b_{560} would remove semiquinone by acting as a Q dismutase in the protonmotive Q cycle, much like cytochrome *b* in complex III; the *kn1* mutation might abolish that function and allow semiquinone to build up. Semiquinone however is notorious for its ability to pass electrons directly to molecular oxygen thus forming superoxide. A problem with this explanation is that complex II has not been demonstrated to be active in a protonmotive Q cycle as yet. The standard redox potential for electron transfer from succinate to CoQ is thought to be insufficient to provide the free energy to drive ATP synthesis (Voet and Voet, 1990). The sensitivity of *mev-1(kn1)* to oxygen could be more reasonably ascribed to the reduced SOD activities, but this phenotype is not discussed in the Ishii et al. (1998) paper.

In very young *mev-1* adults (up to max 48 hours after the L4 to adult molt) oxygen consumption does not differ from that of wild-type animals of the same age (Fig. 2). However, from days 2-7 of adulthood, *mev-1* rates of oxygen consumption are consistently lower than those of wild type (P = 0.030; two-way ANOVA and least significant difference post hoc test). This is consistent with a reduced electron flow from succinate to ubiquinone in *mev-1* mutants (Ishii et al., 1999). Does the dysfunction of MEV-1 succinate dehydrogenase cytochrome

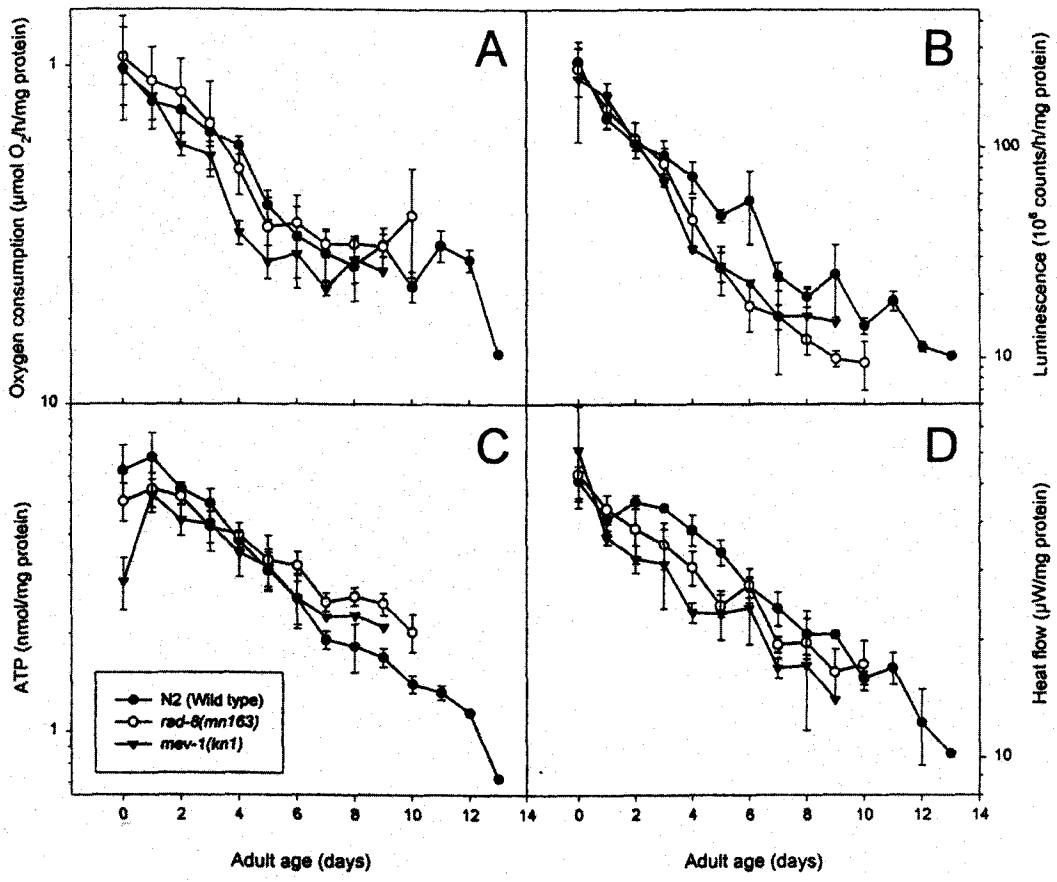

Figure 2: Respiration rates (A), light production potential (B), ATP concentration (C) and metabolic heat output (D) of the wild-type strain N2, and *mev-1(kn1)* and *rad-8(mn163)*.

Newly hatched larvae were grown on agar plates seeded with *E. coli*. Fourth stage larvae were washed off the plates and suspended in 250 ml S buffer containing 7.5 g frozen *E. coli* cells/l and 10 µg/ml cholesterol at worm densities not exceeding 1500/ml. Fluorodeoxyuridine was added at 50 µM final concentration to suppress reproduction. The bacterial cells were added as frozen beads, that had been formed by dripping a 50% suspension of cells into liquid nitrogen and were stored at – 80 °C. The aging cultures were incubated at 24 °C and continuously shaken at 120 oscillations (gyrotory shaker) per min. Bacterial cells were added daily as needed to maintain the initial cell density. At harvest, the worms were cleaned by floatation on 40% (w/w) sucrose (Sulston & Hodgkin, 1988). Dead worms were removed by centrifugation through 36 % Percoll™ (Fabian & Johnson, 1994). Oxygen consumption was measured at 25 °C using a 6 Clark electrode respirometer (Strathkelvin, UK). The light production potential was measured as described in the text. ATP concentration was determined using the luciferin/luciferase assay (Braeckman et al., 1999). Metabolic heat production was measured at 25.0 °C using the Thermal Activity Monitor (Thermometric, Sweden) microcalorimeter, as described in the text.

The study cohorts were initiated simultaneously to reduce environmental variability. The experiment was repeated 3 times; the error bars (SE) represent the variability among the replicate experiments. Time is expressed as adult age; 0 days of adult age indicates that less than 24 h have elapsed since the L4 to adult molt.

b_{560} cause an increase in superoxide levels? The light production assay suggests not. Indeed, *mev-1* mutants can produce similar amounts of light during the first 3 days of their adult lives, but at older ages their capacity to emit light falls below that of wild-type animals of the same age (P = 0.001). In fact, the age-dependent profiles for respiration and for superoxide mediated light production are very similar. *mev-1* mutants initially have lower levels of ATP , relative to wild type (P = 0.004 for days 0-3 of adult life), which is consistent in view of the compromised energy production in these mutants. However, after 3 days of adulthood similar ATP levels are found in *mev-1* and wild type, and at old age (7-9 days of adulthood), wild-type ATP tends to fall below the *mev-1* levels (P = 0.016). We have also monitored the metabolic heat produced in living animals. The nematodes were harvested from the aging cultures that were also used for the respiration, light production and ATP measurements. Sampled worms were washed by sucrose-floatation and suspended in 1 ml of axenic medium. Axenic medium, which can sustain nematode growth, allows heat outputs that are stable for many hours. As measured by its heat output, the *mev-1* mutant is metabolically less active than wild type from day 2 of its adult life onwards ($P << 0.001$) (Fig. 2).

The respiration rate, light production, and metabolic heat profiles suggest that the *mev-1* adults are biologically older than wild type, and this is also supported by the observation that lipofuscin-like material and protein carbonyl derivatives accumulate at an accelerated rate in *mev-1* animals (Hosakawa et al., 1994; Adachi et al., 1998). Again, reduced SOD activity and accelerated

free radical injury are likely implicated, but it remains most unclear as to how *mev-1* is involved. The biological significance of the more gradual decline of ATP in *mev-1* remains unclear.

The activity profiles produced by the *rad-8* mutation are reminiscent of a similar though weaker phenotype, and it will be interesting to learn more about the molecular identity of the *rad-8* gene.

Dietary Restriction may Elicit a Stress Response Program to Increase Longevity

Dietary restriction can substantially increase life span in a number of mammals and it has been demonstrated that it is the reduction of the energetic content that is effective (Masoro, 1988). Calorie-restricted rodents display lower levels of insulin and plasma glucose (Masoro, 1995) and have less propensity to fat synthesis (Feuers et al., 1995). Protein turnover is enhanced in calorie-restricted animals, relative to non restricted animals of the same age, resulting in a more gradual decline of specific enzyme activities (Van Remmen, 1995). In fact dietary restricted animals show signs of delayed aging; they are biologically younger than non-restricted animals of identical age.

There is a widespread belief that caloric restriction may lower metabolic rate. A reduced metabolic rate would then entail less ROS production, resulting in a more gradual aging. Experimental approaches to this hypothesis have yielded equivocal and contradictory results (see recent review in Arking, 1998), however.

The life span of *C. elegans* decreases with increasing bacterial concentration within the range $1x10^8$ (mean life span 25.9 days at 20°C) – $1x10^{10}$ (15.0 days), almost exclusively as a result of shortening of the adult life span (Klass, 1977; Hosono et al., 1989). Lakowski & Hekimi (1998) used a genetic approach to investigate the mechanism of life span extension by caloric restriction. Mutations in *eat* genes reduce the pharyngeal function and result in a decreased feeding rate and a slender appearance. The *eat* mutants live longer and the severity of the Eat and Age phenotypes are generally well correlated which is in keeping with the hypothesis that they live longer as a result of food restriction.

Does the mechanism by which caloric restriction extends life span converge with either the *daf-2/age-1* or *clk* pathway of life span extension? *daf-16(m26)* fails to suppress *eat-2(ad465)* when the slight reduction of life span caused by *daf-16(m26)* is taken into account. In addition, *eat-2(ad465); daf-2(e1370)* double mutants live longer than either single mutant and this is as would be expected if the *eat-2* and *daf-16* genes function in different pathways regulating life span (Lakowski & Hekimi, 1998). *eat-2(ad465);clk-1(e2519)* double mutants display the phenotypes of both mutations, but they do not live longer than either single mutant. This result led Lakowski and Hekimi to believe that caloric restriction (in these experiments represented by *eat-2*) and *clk-1* are involved in a common process of life span extension.

Several observations in axenic culture suggest that the interrelationships of caloric restriction and the *eat* and *clk* mechanisms of life span extension are likely more complex. The generation times in axenic culture for the wild type and most mutant strains tested so far are almost twice as long relative to animals grown on lawns of *E. coli*, but the mean life spans are also 2-fold longer in axenic medium (4 to 5 weeks versus 2 weeks for the wild type, at 24-25°C). Brood size is substantially reduced in axenic culture, and the worms are more slender. These are phenotypes that are also seen when the animals are grown in liquid culture containing low concentrations of bacteria. All these symptoms are reminiscent of dietary restriction. Interestingly, *eat-2* mutant worms grow faster to adulthood, relative to wild type (4.8 ± 0.1 versus 5.7 ± 0.1 days, mean ± SE) and live approximately 40% longer in axenic medium (TABLE 1). *clk-1(e2519)* fails to grow to adulthood in standard axenic medium, but can mature, and lives twice as long as wild type, in axenic medium supplemented with autoclaved *E. coli* cells. In addition, mutation in *daf-16* suppresses the life span extension produced by mutation in *clk-1* by 80% beyond the slight reduction in life span caused by mutation in *daf-16*, suggesting that the *clk-1* and *daf-2/age-1/daf-16* pathways of life span extension converge on the daf-16 gene. In view of the evidence that caloric restriction and the insulin-like pathway lengthen life span by distinct mechanisms (Lakowski & Hekimi, 1998) it can be reasonably assumed that the *clk-1* and *eat-2* pathways of life span extension are also likely distinct from the caloric restriction-specified mechanism.

On the other hand, caloric restriction induces CTL-1, the cytosolic isoform of catalase, in nematodes cultured on limiting amounts of *E. coli* cells (Chalfie, personal communication). We found almost 2-fold higher activity of catalase in axenically cultured animals (unpublished results). These findings are in keeping with the idea that caloric restriction may trigger the activation of the longevity assurance program. This program is specifically activated as L2 larvae are committed to form dauers at the next molt, and is regulated by the insulin-like pathway. These similarities provide suggestive evidence that caloric restriction and the insulin-like pathway converge at some point to elicit a life extending process. By thinking further along these lines we imagine that less insulin-like ligand is produced in axenic medium, resulting in a weaker DAF-2 insulin-like mediated signal, strong enough to promote continued development, but too weak to ensure full repression of the longevity assurance program. At present it is not clear how this model could be possibly reconciled with the conclusions inferred from interaction between the *eat-2*, *clk-1* and *daf-2* genes. A caveat is in order here, however. Firstly, it has not been demonstrated unambiguously that axenic culture medium is a regime of caloric restriction. By caloric restriction, it is generally assumed that the intake of energy is reduced without malnutrition. We are not sure that axenic medium meets this criterion: axenic

medium may impose dietary restriction beyond mere calorie restriction. Secondly, interactions between genes that determine quantitative characters such as life span and reproductive effort is very sensitive to unknown pleiotropic effects which may obscure epistasis analysis.

Similar Correlative Relationships Involving Stress Resistance, Caloric Restriction, Metabolic Rate and Longevity Occur Across Phyla and Kingdoms

Lee et al. (1999) recently analyzed the gene expression profile in skeletal muscle of young adult (5-month-old) and old (30-month-old) mice fed under standard conditions, and 30-month-old mice that received only 76% of the daily caloric intake of the control mice. Aging in non-restricted mice results in an upregulation of stress response genes and lower expression of metabolic and biosynthetic genes. Old calorie restricted (CR) mice have a much better preserved protein turnover. Surprisingly, these mice show a reduced stress response at old age relative to non-restricted mice, at least as suggested by the mRNA profiles. The authors interpret this as a response to reduced metabolic damage, relative to the non-restricted mice. Although several enzymes of the energy metabolism are upregulated in old CR mice, the authors do not conclude that metabolism is possibly upregulated in these animals, relative to the controls. Rather, they state that there is sufficient evidence from other studies to assume that metabolic rate is generally reduced in CR animals, resulting in a lower production of toxic by-products of metabolism. They consider that the CR mediated repression of inducible genes involved in metabolic detoxification, DNA repair, and the response to oxidative stress supports this view, because it would implicate lower substrate availability for these systems. We believe such reasoning is circular. The raw data show a certain degree of ambiguity in the regulation of the energy- versus stress-related genes in old CR mice, but they do not support the idea that CR would reduce metabolic rate.

The authors of this study rightly note that the upregulation of two insulin sensitizer genes in CR mice may witness common mechanisms of life span determination in mice and *C. elegans,* involving insulin-like signaling. Dietary restricted mice have lower levels of both insulin and plasma glucose, suggesting enhanced efficiency of the insulin pathway (Masoro, 1995). Ames dwarf mice live 49% (males) and 64% (females) longer. They are deficient in growth hormone, prolactin and thyroid stimulating hormone, and they produce very low levels of insulin-like growth factor I. Female Ames dwarfs have reduced plasma glucose and insulin levels, much like normal but dietary restricted animals (Bartke et al., 1998; Brown-Borg et al., 1996).

Several groups have selected *D. melanogaster* for long life span by directly selecting for delayed female fecundity. The long-lived stocks obtained are generally resistant to a variety of stress factors including heat or cold stress, oxidative stress, desiccation, ethanol fumes and starvation, and they sustain longer flight (comprehensive review in Arking, 1998). Riha and Luckinbill (1996) found that larvae of their long-lived line underwent caloric restriction without being exposed to any limitation of food supply, i.e. they were committed to feed less actively.

Oxidative stress resistance is likely a major factor determining life span in fruit flies. Strains selected for postponed senescence generally have enhanced activity of antioxidant enzymes (review in Arking, 1998). Transgenic *Drosophila* overexpressing SOD or catalase do not live longer. However, transgenic animals overexpressing both Cu/Zn SOD and catalase live 34% longer than controls (Orr & Sohal, 1994) suggesting that a subtle balance between SOD and catalase is required. In contrast, targeted expression of human SOD1 to *Drosophila* motor neurons increases life span by 40% (Parkes et al., 1998). These cells thus presumably have sufficient catalase activity to neutralize the enhanced production of hydrogen peroxide.

Signal transduction pathways may also modulate stress response and life span in *D. melanogaster*, possibly regulating aging systemically much like in *C. elegans* (Apfeld & Kenyon, 1998). The mutant line *methuselah* (*mth*) lives approx. 35% longer than the parent strain and exhibits enhanced resistance to starvation, high temperature and Paraquat. This phenotype is caused by the insertion of a P-element in the *mth* gene, thereby reducing gene function. The predicted protein is homologous to G protein-coupled transmembrane receptors, raising the possibility that it functions in a signal transduction pathway (Lin et al., 1998).

Stress inducible mechanisms can also extend yeast longevity. For example, mutants resistant to cold and heat stress have postponed senescence under normal conditions. A distinct mechanism that can extend yeast life span is retrograde regulation. This is a sequence of signaling events resulting in the selective induction of nuclear genes. The final outcome is a shift of metabolic activities, including potentiation of the glyoxylate cycle. A similar sequence of events accompanies sporulation following caloric stress. Both life span extending pathways are linked by the involvement of RAS2, which participates in sensing the nutritional status and in responding to stress as well. The long-lived yeast cells acquire increased metabolic capacity and efficiency (Jazwinski, 1996; 1999; Sun et al., 1994).

A stress-induced response, triggered in stationary-phase *E. coli* culture, increases cell survival during stasis. An initial increase in oxidation of specific target proteins elicits the induction of the heat shock regulon, which protects the aging cells against oxidative stress and thermal stress (Dukan and Nyström, 1998).

CONCLUSIONS

It seems safe to conclude that the role of innate anti-stress programs in extending life is too widespread

among organisms to be purely coincidental. Stress response programs that would allow survival under harsh conditions and delay fertility would offer a selective advantage in an unpredictable environment, and likely evolved early in evolution. The life-extending mutations generally delay the onset of the aging process but do not alter the age-related increases of mortality appreciably. This is apparent from the survival curves obtained for *C. elegans*: they slide a distance over the time axis (to the left in short-lived mutants, or to the right in long-lived mutants) corresponding with the severity of the mutation, while the shape of the curve remains essentially unchanged (Vanfleteren et al., 1998b). The survival curves that have been published for yeast, *D. melanogaster* and mammals generally follow this pattern. This could mean that the longevity genes identified as yet can delay or advance the onset of aging, but do not specify the aging process. Since modulation of the antioxidant enzyme activities is the most common final output of these genes, cumulative damage by ROS is likely a major component of the aging process. In this context we further speculate that these longevity genes fortuitously use the devastating action of ROS to determine life span by adjusting repair and prevention to the needs imposed by natural selection.

The relation of metabolic activity and life span remains elusive. Metabolic homeostasis may deteriorate with aging as a result of ROS-derived damage. Alternatively, metabolic activity, particularly protein turnover, might be increasingly repressed as a function of age by longevity determining genes. This model would imply a selective advantage earlier in life, possibly linked to the optimal allocation of resources to reproduction and maintenance, as suggested by Kirkwood & Rose (1991).

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